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Comparison of autotrophic and mixotrophic cultivation of green microalgal for biodiesel production

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Abstract

The effects of autotrophic and mixotrophic growth on cell growth and lipid productivity of green microalgae *Chodatella* sp. were investigated. Carbon dioxide and piggery wastewater served as the carbon and nutrient sources, respectively, for autotrophic and mixotrophic growth. Appropriate doses of each source were found to be beneficial to biomass production. The cultures produced similar fatty acid compositions, which are suitable for biodiesel production. The specific growth rate, biomass production, and lipid productivity obtained with mixotrophic growth were 1.74, 14, and 5.6 times higher than those obtained with autotrophic growth, respectively. The mixotrophic cultivation simultaneously assimilated 99.7% ammonia nitrogen and 75.9% total phosphorus from piggery wastewater, which reduced the required nutrient for the culture of microalgae, thereby reducing the cost of biodiesel production.

Keywords: autotrophic, biodiesel production, mixotrophic

Introduction

Despite many microalgae being exceedingly rich in lipid content as biodiesel feedstock, limitations such as efficacy and cost-competitiveness must be addressed [1, 2]. One of the methods used for reducing the cost of algal biomass is to integrate wastewater treatment with algae biomass production [3]. For example, Lau et al. [4] reported that over 90% of N content and 80% of P content were removed by *Chlorella vulgaris* from primary treated sewage. A similar microalgal strain, *Chlorella pyrenoidosa*, in soybean processing wastewater was demonstrated not only to remove 78% of soluble organic carbon, 89% of total nitrogen (TN), and 70% of total phosphate (TP), but also attained an average biomass productivity of 0.64 g/L·d with an average lipid content of 37% [5]. Sydney et al. [6] found that *Botryococcus braunii* is able to remove N and P nutrients (79.63%) from treated domestic wastewater, and accumulate oil with a dry biomass of up to 36%. The lipid profiles of this extracted oil are similar to those of oilseed feedstocks.

The accumulation of lipids and the cell growth rate in wastewater depend on diverse factors, such as cultivation conditions, algal species, and growth environment [1,3]. An excessive nutrient concentration can inhibit algal growth [7,8]. The organic carbon/nitrogen (C/N) ratio and the carbon/phosphorous (C/P) ratio in wastewater may be low compared to the typical ratios in rapidly growing algal biomass (C/N 6:1; C/P 48:1) [8]. Li et al. [9] found that at a 5-8:1 N/P ratio, both nutrients can be highly removed by *Scenedesmus* sp. in an artificial medium. Li et al. [9] also found that N or P limitation can lead to the accumulation of lipids (30% vs. 53%). However, there is a trade off between maximal algal lipid content and optimal algal growth in wastewater, the former requiring low nutrient content and the latter requiring high organic nutrient concentration [10]. A major limitation is that despite inducing very high lipid content, the cell growth rate is often very low, which limits lipid production [11,12].

Swine breeding is an important industry in Taiwan, with piggery wastewater discharged from over 6 millions pigs. Algal growth in wastewater that contributes to renewable energy production and wastewater treatment is a promising strategy. However, there is little information available for cultivating microalgae in a piggery wastewater environment. The strain of *Chodatella* sp. isolated from local fresh water was found to grow in both autotrophic and mixotrophic modes. The present study compares the cell growth rate and biodiesel production under autotrophic and mixotrophic growth.

Materials and methods

2.1 Microalgal culturing

One of the dominant green microalgal species, *Chodatella sp*., was isolated from local source water and cultured in a medium according to the method presented by Norris et al. [13]. Autotrophic cultures of *Chodatella sp*. were grown in batch mode in a 5-L modified serum bottle containing 4 L of sterilized algal medium. The bottles were placed in an incubator maintained at 25 °C and continuously provided with 5 klux of illumination. CO_2 was supplied to the cultures by syringe injection every day. For mixotrophic growth, cells were incubated with piggery wastewater at 25 °C with continuous illumination of 5 klux and aeration with air at a 0.8 L/min·L flow rate. The piggery wastewater effluent was collected from a local pig farm at Pingtung University of Science and Technology, Taiwan. The piggery wastewater was filtered through a 0.45-μm membrane and sterilized before experiments. Cultures were harvested in the log growth phase for experiments. For autotrophic cultures,

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the CO_2 concentrations of the medium were 2, 4, and 8% (vol.), and the piggery wastewater content were 20, 50, and 100% for mixotrophic cultures, respectively.

2.2. Biomass concentration and growth rate

The optical density of microalgal cells was determined by measuring absorbance at 684 nm (OD_{684}) using an ultraviolet/visible spectrophotometer (Model U-2001, Hitachi, Japan). The dry weight of the microalgal biomass was determined gravimetrically. A known volume of microalgal culture was collected and dried at 90 °C for 3 hours. The growth rate (μ) was calculated according to the equation μ = (ln A1 - ln A0)/(T1 - T0), where A1 and A0 are the dry weights of the microalgal biomass at times T1 and T0, respectively. The relationship between microalgal biomass and OD_{684} is: biomass = 14.447 + 259.678 OD_{684} (R^2 = 0.972, P < 0.0001).

2.3. Lipid content

A stock culture of *Chodatella* sp. cells was collected by centrifugation at 5000 × g for 5 min. The precipitated algal cells were washed and resuspended in deionized water in triplicate. Cells were collected by centrifugation again and then dried by a freeze dryer. The microalgal total lipids were extracted with n-hexane/methanol (2/1, v/v) in a Soxhlet extractor and quantified gravimetrically. The biomass concentration (mg/L) is expressed as the dry weight of the microalgal biomass.

2.4. Fatty acid methyl esters

Freeze-dried biomass (0.1 g) was placed in 50-mL Teflon-capped Pyrex tubes and mixed with a premixed homogeneous solution of NaOH catalyst (2.5 wt.%) and methanol. 8.0 mL of alkali catalyst was added to the tubes. The reaction mixture was heated at 60 °C for 30 min, with the samples well-mixed during heating. After the reaction was completed, the tubes were allowed to cool to room temperature. Then, 8 mL of an acid catalyst (HCl:methanol, 5.8 vol.%) and 10 mL of a boron trifluoride/methanol solution were added to the tubes in a water bath at 100 °C for 15 min. After the reaction was completed, the tubes were allowed to cool to room temperature. Purification of the solution was achieved by using 2 mL of saturated sodium chloride solution. 4 mL of an extracting solvent (hexane) was then added to each tube. The upper solvent layer containing fatty acid methyl esters (FAMEs) was collected and analyzed by gas chromatography.

FAME analysis was performed using a gas chromatograph with an autosampler (Agilent 7820A, USA), flame ionization detector, and a DB-23 Agilent column (length: 60 m, ID: 0.25 mm, film: 0.25 μm). Hydrogen was used as the carrier gas at a constant flow rate of 35 mL/min. The injector was held at 270 °C while the detector was kept at 280 °C. The oven was at 130 °C initially, ramped to 170 °C at 6.5 °C/min, then ramped again to 215 °C at 2.75 °C/min, and held at 215 °C for 12 min. The temperature was further increased to 230 °C at a heating rate of 40 °C/min and held for 3 min, giving a total heating time of 38.88 min. FAMEs were identified by comparing their retention times with those of standard fatty acids (Supelco, USA) and quantified by comparison with the prepared calibration curves. Pentadecanoic acid (C15:0) was used as an internal standard.

2.5. Nitrogen and phosphorus analysis

The samples were first filtered through a 0.45-μm membrane and then the filtrate was properly diluted and analyzed for ammonia nitrogen (NH_3-N), organic nitrogen, and orthophosphate (PO_4^{3-}-P). NH_3-N and PO_4^{3-}-P concentrations were determined according to Standard Methods [14]. Total Kjeldahl nitrogen (TKN), the sum of NH_3-N and organic nitrogen, was measured in the experiments.

RESULTS AND DISCUSSION

3.1 Effect on cell growth

The cell growth indicated by optical density for various CO_2 and piggery wastewater content levels is shown in Fig. 1. It can be seen that *Chodatella* sp. survived in all of the cultures and no obvious lag phases were observed except for the culture under 100% piggery wastewater content. A comparison of the cultures shows that cells grew faster under piggery wastewater cultivation than under CO_2 cultivation. For example, the optical density of the cells was 1.87 cm^{-1} at 100% piggery wastewater content in the 7th day. Under CO_2 cultivation, the optical density of the cells reached 0.693 cm^{-1} at 4% content in the same cultivation period. The average specific growth rates under cultures with piggery wastewater content levels of 20, 50, and 100% were 0.24, 0.281, and 0.303 d^{-1}, respectively. These specific growth rates are higher than those under CO_2 cultivation (0.14, 0.173, and 0.114 d^{-1} for CO_2 concentrations of 2, 4, and 8%, respectively).

Several factors influence algal cell growth, including the availability of nutrients, light (quality and quantity), pH, temperature, and the initial inoculation density. Figure 1a shows the growth curve under CO_2 cultivation. The optimal CO_2 content for cell growth is 4%. Insufficient CO_2 concentration (2%) in the culture would not sustain optimal growth. However, higher CO_2 concentration did not further improve cell growth Similar results were reported for *Scenedesmus obliquus* by Kaewkannetra et al. [15]. This is probably due to excess CO_2 being converted to H_2CO_3, resulting in a reduction of pH in the culture, thereby affecting cell growth. Greque de Morais and Costa [16] reported that a *Spirulina* sp. culture with 6% CO_2 had the maximum specific growth rate. Higher CO_2 concentration led to a decrease of biomass yield.

Under piggery wastewater cultivation, the optimal content for cell growth is 50% (Fig. 1b). With 100% piggery wastewater content, the culture had a lag phase during the first 2 days. It is speculated that this may be due to the relatively high ratio of nutrient concentration to initial inoculation density. Cell growth dramatically increased after 4 days under 100% piggery wastewater content. This mixotrophic culture produced a 1.74 times

higher specific growth rate compared with that for autotrophic growth with 4% CO_2 content.

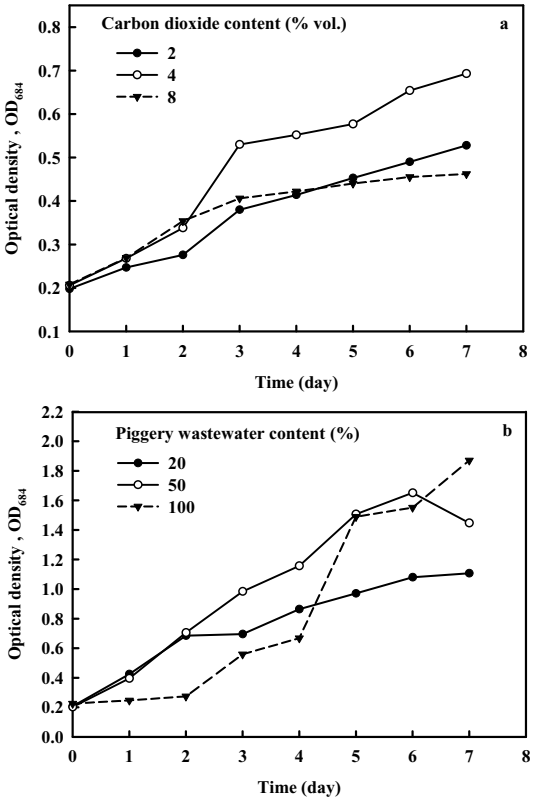


Fig. 1. Cell growth in various content of (a) CO_2 and (b) piggery wastewater cultivation

3.2 Effect on biomass and lipid productivity

Fig. 2 shows the effects of CO_2 and piggery wastewater cultivation on biomass and lipid productivity. Although both CO_2 and piggery wastewater cultivations could sustain microalgal growth, the biomass and lipid productivity obtained with piggery wastewater were much higher than those obtained with CO_2. Even with the lowest productivity for piggery wastewater cultivation (20% content), biomass and lipid productivity were higher than those obtained with the optimal CO_2 content (4%). The biomass productivity (Fig. 2c) reached up to 115 mg/L·d under 50% piggery wastewater content, whereas that with cultivation with 4% CO_2 content was only 28.6 mg/L·d (Fig. 2a). Biomass production and the associated cell growth, as demonstrated by biomass productivity, indicate that mixotrophic growth with piggery wastewater is more beneficial than autotrophic growth. This result is similar to the findings for *Chlorella vulgaris* [17] and *Chlorella protothecoides* [18], where mixotrophic cultures with glucose or acetate had higher biomass productivities than those of autotrophic cultures.

Lipid productivity is the mass of lipids produced per unit volume of the culture per unit time; it depends on the algal growth rate and the lipid content of the biomass. Fig. 2b shows the effect of CO_2 content on lipid productivity. The highest lipid productivity was achieved with 4% CO_2 content, implying that there exists an optimal dosage for CO_2 cultivation. This finding is consistent with the biomass productivity data. However, 8% CO_2 content resulted in a lower lipid content in cells (data not shown); therefore, a decrease in lipid productivity is more significant than that of biomass productivity. The results show that high CO_2 content not only decreased cell growth, but also significantly hindered lipid accumulation.

Under piggery wastewater cultivation, the biomass productivity increased when the content was higher than 50%. The culture with 100% wastewater produced a lower lipid content than that produced by the culture with 50% wastewater (Fig. 2d), and thus had lower lipid productivity. The 50% piggery wastewater content culture reached 51.4 mg/L·d lipid productivity, approximately 14 times higher than that obtained with the culture with 4% CO_2 content. The better performance of mixotrophic growth with piggery wastewater compared to that of autotrophic growth is conjectured to be due to the homogeneous distribution of nutrients and favorable carbon utilization in mixotrophic culture. This lipid productivity is much higher than that reported by Wang [19] for *Chlorella pyrenoidosa* cultivated in piggery wastewater (maximum lipid productivity of 6.3 mg/L·d).

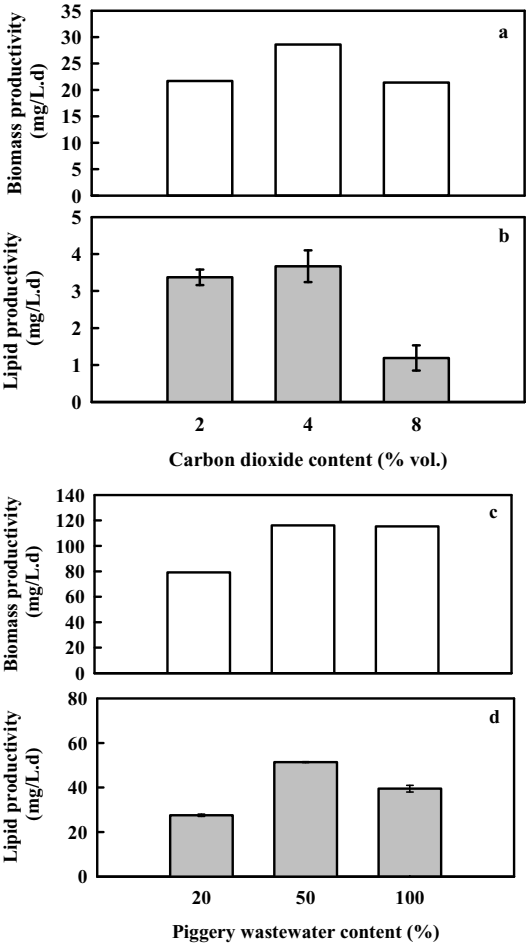
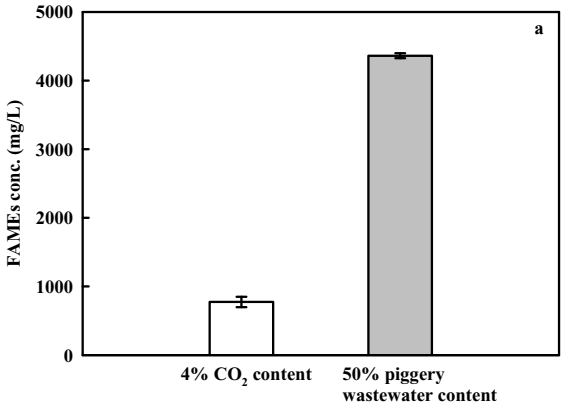


Fig. 2. Biomass and lipid productivity in various content of (a, b) CO_2 and (c,d) piggery wastewater cultivation

3.3 Effect on fatty acid content and composition

Fig. 3 compares the FAME concentration and FAME composition of cultures with the optimal levels of CO_2 and piggery wastewater. Similar to lipid productivity, the amount of total FAMEs produced from piggery wastewater cultivation was much higher than that obtained with CO_2 cultivation. Under the testing conditions, cultivation with 4% CO_2 and 50% piggery wastewater produced similar fatty acid compositions, comprising mainly palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). Oleic acid (C18:1) was the most abundant fatty acid in algal cells under piggery wastewater cultivation. Palmitic acid (C16:0) was the second most abundant fatty acid. Palmitic and oleic and fatty acids were also the most abundant fatty acids for CO_2 cultivation.

These four major fatty acids produced from autotrophic CO_2 growth and mixotrophic piggery wastewater growth are quite similar to those of soybean oil and rapeseed oil and are thus suitable for biodiesel production. However, the total amount of FAMEs obtained with mixotrophic growth was 5.6 times higher than that obtained with autotrophic growth. This indicates that lipids produced by microalgae under mixotrophic growth have great potential as economical feedstock for biodiesel production, due to the fact that piggery wastewater can serve the essential nutrients and carbon supply for algae growth. Sturm et al. [20] confirmed that algal biofuel production is energetically favorable when utilizing wastewater as a nutrient source. Mixotrophic growth produces algal biomass while removing nutrients from wastewater, making it a feasible and promising strategy [21].



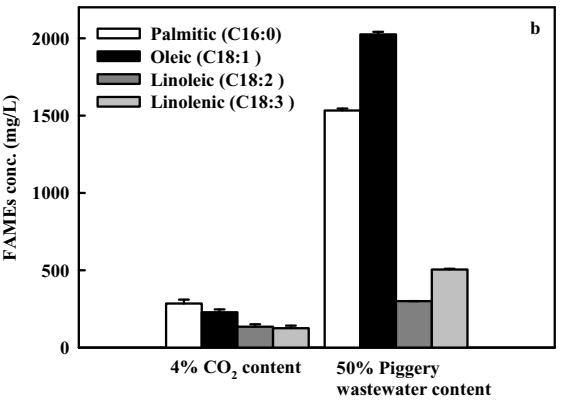


Fig. 3. Fatty acid concentration and compositions of (a) CO_2 and (b) piggery wastewater cultivation

3.4 Biomass production with nutrient removal

Fig. 4 shows the changes in TN, NH_3-N, TKN, PO_4^{3-}-P, biomass concentration, and cell lipid content with time for cultivation with 50% piggery wastewater. Ammonium decreased from 44.83 mg/L to 0.16 mg/L and TKN dropped from 69.04 mg/L to 40.99 mg/L after 12 days of cultivation (Fig. 4a). The total phosphorus drastically decreased from 2.79 mg/L to 0.67 mg/L in the same period (Fig. 4b). The removal efficiencies of TKN, NH_3-N, and TP are 48.6%, 99.7%, and 75.9%, respectively. As mentioned above, TKN is the sum of NH_3-N and organic nitrogen. Organic nitrogen increased after cell growth (data not shown) due to the release of cell endogenous decay. Therefore, ammonium is main form of nitrogen to provide sufficient nutrient for meeting the cell growth in piggery wastewater [22].

The decrease in nitrogen and phosphorus resulted in an increase in biomass concentration (Fig. 4c). The biomass concentration increased from 136 mg/L to 1,406 mg/L after 12 days. The cell lipid content also increased with cultivation time, up to 41.2% at the 12th day. Numerous researchers have reported that algal cell lipids accumulate under nutrient limitation in the culture [23-25]. However, nutrient limitation inhibits cell growth, and thus lowers lipid productivity. In this case, no significant reduction of biomass production was observed even under lower NH_3-N and TP concentrations after the 4th day. Specific nutrient removal rates of 3.72 mg N/L·d and 0.18 mg P/L·d were obtained. These results are consistent with the nitrogen and phosphorous removal rates reported by Ruiz et al. [26] for a culture of freshwater *Scenedesmus obliquus* in a synthetic medium (removal rates of 13.5-4.2 mg N/L·d and 1.49-0.32 mg P/L·d).

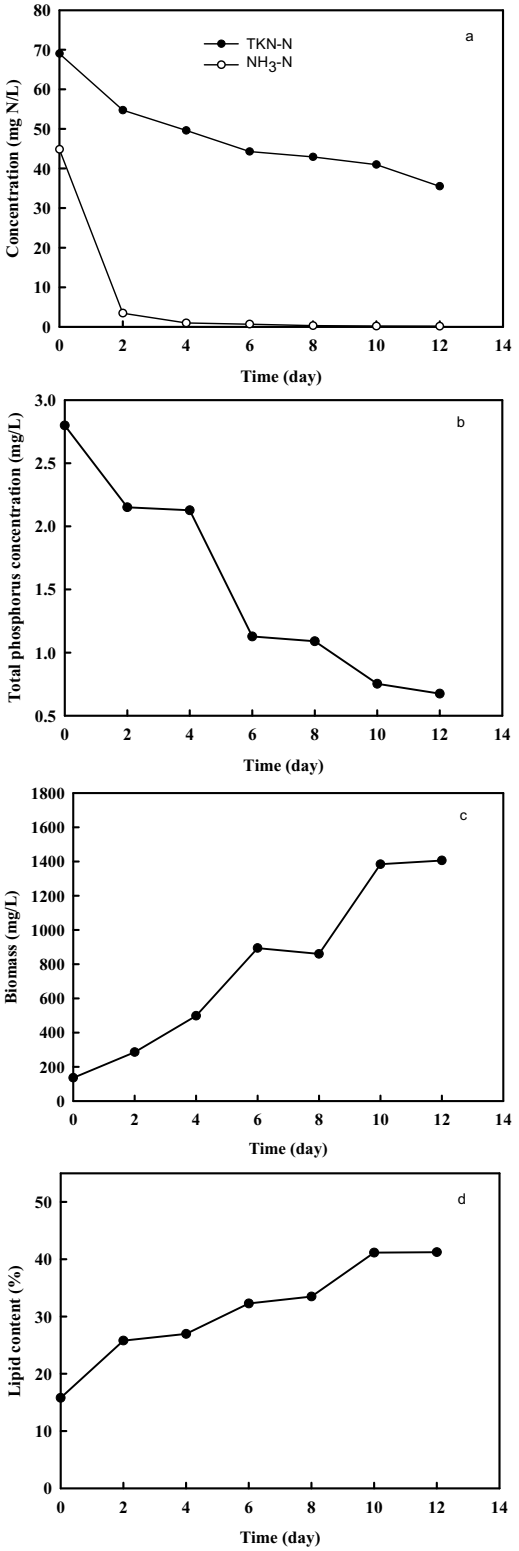


Fig. 4. (a) Nitrogen concentration, (b) total phosphorus concentration, (c) biomass concentration, and (d) cell lipid content during batch culture on 50% piggery wastewater

Conclusion

The effects of cultivation conditions on algal biomass and lipid production of green microalgae (*Chodatella* sp.) grown in autotrophic and mixotrophic modes were investigated. Carbon dioxide and piggery wastewater served as the carbon and nutrient sources, respectively. Under both autotrophic and mixotrophic cultivation conditions, an appropriate dose of each source was beneficial to biomass production. Both cultivation method led to similar fatty acid compositions, comprising palmitic (C16:0), oleic (C18:1), linoleic (C18:2), and linolenic (18:3) acids. The specific growth rate, biomass production, and FAME yield obtained with mixotrophic growth were much higher than those obtained with autotrophic growth. *Chodatella* sp. is a potential strain for integrating piggery wastewater treatment with algae biomass production. Its mixotrophic growth produced biodiesel practically and economically.

Acknowledgment

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